The use of non-methylated DNA as a control and calibration standard

Background of the invention.

The present invention relates to the use of DNA, in which 5-methylcytosine does not occur. Such non-methylated DNA is in particular required as a verification for a reliable and sensitive analysis of cytosine methylations.

5-methylcytosine is the most frequent covalently modified base in the DNA of eukaryotic cells. It plays an important biological role, amongst others for the transcription regulation, for the genetic imprinting and in the tumor genesis (for a survey: Millar et al.: Five not four: History and significance of the fifth base. In: The Epigenome, S. Beck and A. Olek (eds.), Wiley-VCH Verlag Weinheim 2003, pages 3 - 20). The identification of 5-methylcytosine as a component of genetic information is therefore of enormous interest. A detection of the methylation is however difficult, since cytosine and 5-methylcytosine have the same base pairing behavior. Many of the conventional detection methods based on hybridization can therefore not distinguish between cytosine and methylcytosine. Further, the methylation information gets completely lost with a PCR amplification.

The conventional methods for the methylation

analysis are substantially based on two different principles. On the one hand, methylation-specific restriction enzymes are used; on the other hand, a selective chemical conversion of non-methylated cytosines into uracil (so-called bisulphite treatment, see for instance: DE 101 54 317 A1; DE 100 29 915 A1) takes place. The enzymatically or chemically pretreated DNA is then in most cases amplified and can be analyzed in different ways (for a survey: WO 02/072880 p. 1ff). Of great interest are methods, which are capable to sensitively and quantitatively detect methylation. Due to the important role of the cytosine methylation in the occurrence of cancer, this applies in particular with regard to diagnostic applications. Up to now, the conventional methods secure a sensitive and quantitative methylation analysis to a limited degree only.

For the sensitive analysis, the chemically pretreated DNA is usually amplified by means of a PCR method. By the use of methylation-specific primers or blockers, then a selective amplification only of the methylated (or in the reverse reaction: non-methylated) DNA is secured. The use of methylation-specific primers is known as the so-called "methylation-specific PCR" ("MSP"; Herman et al.: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA. 1996 Sep 3; 93(18):9821-6). Thereby is achieved in particular the qualitative detection of methylated DNA having a low concentration. A comparably sensitive method is the so-called "HeavyMethyl" method. Therein, a specific amplification only of the originally methylated (or non-methylated, resp.) DNA is achieved by the use of non-methylation-specific

blocker oligomers (i.e. blocker oligomers, which hybridize at converted, originally non-methylated nucleic acids; for a survey: WO 02/072880; Cottrell et al.: A real-time PCR assay for DNA-methylation using methylation-specific blockers. Nucl. Acids. Res. 2004 32:e10). MSP as well as HeavyMethyl can be used as quantifiable "real time variants". They permit the detection of the methylation status of positions immediately in the course of the PCR, without a subsequent analysis of the products being required ("MethyLight" - WO 00/70090; US 6,331,393).

However, a reliable quantification of the methylation status over a linear range by the above methods is possible up to now to a limited degree only. For this, it is necessary that the assays are calibrated with fully methylated as well as with non-methylated DNA (cf.: Trinh et al.: DNA methylation analysis by MethyLight technology. Methods. 2001 Dec; 25(4): 456-62). The production of fully methylated DNA is relatively simple by the use of the SssI methylase. This enzyme transforms in the sequence context 5'-CG-3' all non-methylated cytosines into 5-methylcytosine. Problematic, however, is the production of fully non-methylated DNA. An enzyme corresponding to the SssI methylase, which quantitatively removes all methyl groups, is not available. Up to now, sperm DNA having a low methylation degree is used for the calibration (cf.: Trinh et al. 2001, ibid.). However, the sperm DNA is partially methylated and can thus to a limited degree only be used as a reliable standard. Further, artificially produced, short non-methylated sequences such as PCR amplificates can also to a limited degree only be used, for instance for the analysis of individual de-

fined positions. For multiplex reactions, these standards cannot be used, since the complexity of the reaction would then be too high. Further, the development of every new detection assay requires the production of a new defined standard. In contrast, a non-methylated standard covering the complete genomic DNA or a representative part thereof would permit a reliably quantifiable methylation analysis. Further, a standardized and thus simple, cost-effective and quick development of new detection assays would be possible. Because of the specific biological and medical importance of the cytosine methylation and because of the drawbacks mentioned above of the standards used today, there is a great technical need of methods, which make the genomic DNA in a fully non-methylated form available. In the following, such a - surprisingly simple - method is described.

According to the invention, so-called genome-wide amplification methods (WGA - whole genome amplification, for a survey: Hawkins et al.: Whole genome amplification - applications and advances. Curr Opin Biotechnol. 2002 Feb; 13(1): 65-7) are used for the production of non-methylated DNA. In this method, a large part of the genomic DNA is multiplied by means of a DNA polymerase and "random" or degenerated primers. "Random" primers are such primers, which do not specifically bind to certain nucleic acids, but to a multitude of nucleic acids. Thereto belong primers, which are either very short (between 5 and 10 bp), or primers, which are called "degenerated primers". Such degenerated primers are primers, which do not specifically bind to certain nucleic acids, since they contain either universal bases, which bind to several different

nucleotides, or a mixture of primers is used, which differ in the "degenerated" positions. Universal bases are bases, which bind to several different nucleotides (see e.g. Promega catalog: Pyrimidine or purine-specific universal bases). Both are understood in the following as "degenerated primers". In the amplifications, only non-methylated cytosine triphosphates are offered, so that the amplificates are synthesized fully non-methylated. After several amplification runs, the amount of the partially methylated matrix DNA is fully in the background, compared to the newly produced, non-methylated nucleic acids.

Up to now, different WGA methods have been described. In the so-called primer extension preamplification (PEP), the amplification is performed by means of a Taq polymerase and a random mixture of oligonucleotide primers with a length of approx. 15 nucleotides (Zhang et al.: Whole genome amplification from a single cell: implications for genetic analysis. Proc Natl Acad Sci USA. 1992 Jul 1; 89(13): 5847-51}. In the DOP-PCR (degenerate oligonucleotide primed polymerase chain reaction), however, one degenerated primer only is used (cf.: Telenius et al.: Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics. 1992 Jul; 13(3): 718-25). Another WGA method is the so-called linker adaptor PCR. Therein, the DNA is first digested by means of a restriction enzyme. Then, linkers are ligated at the restriction fragments. In the following amplification, primers are used, which specifically bind to the linkers (for a survey: Cheung and Nelson: Whole genome amplification using a degenerate oligonucleotide primer allows

hundreds of genotypes to be performed on less than one nanogram of genomic DNA. Proc Natl Acad Sci USA. 1996 Dec 10; 93(25): 14676-9 with other documents). The PCR-based WGA methods described above have, however, several drawbacks. For instance, the generation of unspecific amplification artifacts may happen. Further, all genome sections are only incompletely covered. Moreover, there are created in part short DNA fragments having a length of less than 1 kB. (cf.: Dean et al.: Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA. 2002 Apr 16; 99(8): 5261-6 with other documents). The at present most effective method for the genome-wide amplification is thus the isothermal "multiple displacement amplification" (MDA, cf.: Dean et al. 2002 ibid.; US Patent 6,124,120). Therein, the genomic DNA is reacted with "random" primers and a DNA polymerase. Polymerases are used, which are capable to drive the non-template strand of the DNA double strand during the amplification out (for instance a φ29 polymerase). The driven-out strands in turn serve as a matrix for the extension of further primers. By this method it is possible to produce approx. 20-30 µg DNA from 1 - 10 copies only of human genomic DNA. This corresponds to a more than 5,000-fold amplification. The average product length is more than 10 kB, and the amplification occurs rather uniformly over the complete genome. The reaction may take place directly from biological samples, for instance from blood or cell cultures. Commercially available kits for the MDA are at present offered by two suppliers ("GenomiPhi" of Amersham Biosciences, www.4amersham biosciences.com; "Repli-g" of Molecular Staging, www.molecularstaging.com). DNA already amplified

is also available from these suppliers. The DNA produced by means of MDA is used in a great variety of applications, for instance in the genotyping of single nucleotide polymorphisms (SNP), in the "chromosome painting", in the restriction fragment length polymorphism analysis, in the subcloning and in the DNA sequentiation. The MDA can thus be used in particular for genetic, forensic and diagnostic investigations (cf.: Dean et al. 2002, ibid.).

The use of DNA produced by WGA methods as a standard in methods for the detection of 5-methylcytosine is not yet known up to now. The applications described in more detail in the following therefore allow the methylation analysis to have for the first time access to genomic, non-methylated DNA. Due to the special importance of the cytosine methylation and due to described drawbacks of the prior art, this advantageous, new technique is an essential technical progress.

Specification

According to the invention, the DNA produced by genome-wide amplification methods is used as a standard in the methylation analysis. According to the invention is further provided a method for the methylation analysis, which is characterized by that

a) a genome-wide amplification is performed,

b) the amplificates received therefrom are used in the methylation analysis as a standard.

In principle, according to the invention, all WGA methods described above can be used. The reaction conditions of the PEP, DOP-PCR and linker-PCR also belong to the state of the art (see above). Because of the drawbacks of the PCR-based WGA methods, according to the invention an MDA is preferably performed. The reaction conditions for an MDA method are also sufficiently known (cf.: Dean et al 2002, ibid.; US patents 6,124,120; 6,280,949; 6,642,034; US application 20030143536; product information about the Genomiphi and Repli-g kits mentioned above). Other variations of the WGA, too, in particular of the MDA method, can be used according to the invention for the production of non-methylated DNA. For instance, it is possible to first fragment the DNA and to ligate linkers at the fragments. Subsequently, the fragments are transferred into concatamers, which are then amplified by means of an MDA (multiple strand displacement amplification of concatenated DNA MDA-CA; cf.: US 6,124,120).

According to the invention it is preferred to use a conventional MDA, however. Preferably, two sets of primers are used. One primer set respectively is complementary to one strand of the DNA to be amplified. The primer sets may be random primers or degenerated primers. Details with regard to the number, length and structure of the primers have often been described (cf.: US 6,124,120). For instance, it is known that primers can be used, which are at the 5' end not complementary to the target sequence. Thereby, the driving-out of the primers by the polymerase is facilitated. The 5' region of the primers may in addition carry functional sequences, for instance for a promoter (cf.: US 6,124,120). The

optimum structure of the primers depends on the type of the used polymerase, in particular on its processivity (cf.: US 6,124,120). Particularly preferred are hexamer primers. Different polymerases can be used in the MDA reaction. The enzymes must be capable either alone or in combination with auxiliary factors (for instance helicases) to drive the non-matrix strand of the DNA double helix to be replicated during replication out. For this, polymerases are preferred, which do not have a 5'-3' exonuclease activity. Alternatively, primers can also be used, which are blocked at the 5' end, and are therefore not degradable by the polymerases. As a polymerase, the φ29 polymerase is particularly preferred. The latter has a very high processivity permitting to synthesize DNA very effectively, even when extreme base compositions, short tandem repeats or secondary structures occur in the DNA. In the US patent 6,124,120 and in the US patent application 2003/0143536 A1, further possible polymerases are mentioned, such as Bst, Bca or phage M2-DNA polymerase. The reaction conditions required for the amplification depend on the selection of the polymerases and the primers and also follow from the references named above. It is also known, amongst others, that a detection and quantification of the amplified DNA can be obtained by various methods, for instance by the incorporation of marked nucleotides, by the use of special detection probes or by solid phase detectors (cf.: 6,124,120).

In a preferred embodiment of the invention, the commercially available kits are used for the synthesis of the non-methylated DNA. Particularly preferred are the kits "GenomiPhi" (Amersham Biosciences) or "Repli-g" (Molecular Stag-

ing). The amplification takes place according to supplier's instructions. Basically, the DNA to be amplified is reacted with a sample buffer and random hexamer primers. The mixture is heat-denaturated and then cooled down, so that a binding of the primers to the DNA can occur. Then, the remaining reaction components, in particular the desoxynucleoside triphosphates and the φ29 polymerase are added. The reaction mixture is then incubated for approx. 30 hours at 30°C. As an initial material, for instance DNA can be used, which has been isolated by the commercially available purification methods. For cellular samples such as blood samples or primary cells from clinical samples, an alkaline lysis with subsequent neutralization may be sufficient (cf.: product information of Amersham for the GenomiPhi DNA amplification kit).

In a particularly preferred embodiment of the invention, commercially available DNA produced by means of MDA (see above) is used as a standard. This has the advantage that the DNA has a constant concentration and quality because of the standardized production processes.

The DNA produced by using the above methods or commercially acquired can be used as a standard in a multitude of methylation analysis methods. Thereto belong methods based on the use of restriction enzymes as well as methods based on a bisulphite treatment of the DNA (cf.: Fraga and Esteller: DNA Methylation: A Profile of Methods and Applications. Biotechniques 33:632-649, September 2002). Preferably, first a bisulphite conversion is performed. The bisulphite conversion is known to the man skilled in the art in different variations (see for instance:

Frommer et al.: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA. 1992 Mar 1; 89(5): 1827-31; Olek, A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Res. 1996 Dec 15; 24(24): 5064-6; DE 100 29 915; DE 100 29 915). It is particularly preferred that the bisulphite conversion is made in presence of denaturating solvents, such as dioxane, and a radical catcher (cf.: DE 100 29 915). In another preferred embodiment, the DNA is converted not chemically, but enzymatically. This is for instance imaginable by using cytidine deaminases, which react non-methylated cytidines more quickly than methylated cytidines. A respective enzyme has been identified just recently (Bransteitter et al.: Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. Proc Natl Acad Sci USA. 2003 Apr 1; 100(7):4102-7).

The converted DNA can be analyzed by means of conventional molecular biological methods, such as hybridization or sequentiation. In a preferred variant, the converted DNA is first amplified. For doing this, the man skilled in the art is familiar with different methods, such as ligase chain reactions. Preferably, the DNA is however amplified by a polymerase reaction. Various modifications are imaginable for this, for instance the use of isothermal amplification methods. Particularly preferred are however polymerase chain reactions (PCR). In a most particularly preferred embodiment, the PCR is performed by using primers, which specifically bind to positions of the converted sequence only,

which were either methylated before (or in the reverse reaction: non-methylated) (MSP, see above). In another most particularly preferred embodiment, the converted DNA is analyzed by means of methylation or non-methylation-specific blockers ("HeavyMethyl" method, see above). The detection of the PCR amplificates may be made by conventional methods, for instance by methods of the length measurement such as gel electrophoresis, capillary gel electrophoresis and chromatography (e.g. HPLC). Mass spectrometry and methods for the sequentiation such as the Sanger method, the Maxam-Gilbert method and sequencing by hybridization (SBH) may also be used. In a preferred embodiment, the amplificates are detected by primer extension methods or by methylation-specific ligation methods (see for instance: Gonzalgo & Jones: Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (MsSNuPE). Nucleic Acids Res. 1997 Jun 15; 25(12): 2529-31; DE 100 10 282; DE 100 10 280). In another preferred embodiment, the amplificates are analyzed by means of hybridization at oligomer microarrays (cf.: Adorjan et al.: Tumor class prediction and discovery by microarray-based DNA methylation analysis. Nucleic Acids Res. 2002 Mar 1; 30(5): e21). In another particularly preferred embodiment, the amplificates are analyzed by using PCR real time variants (cf.: Heid et al.: Real time quantitative PCR. Genome Res. 1996 Oct; 6(10): 986-94, US patent No. 6,331,393 "MethyLight"). Therein, the amplification is performed in presence of a methylation-specific, fluorescence-marked reporter oligonucleotide. The reporter oligonucleotide then preferably binds to the DNA to be investigated and indicates the amplification thereof by

increase or decrease of the fluorescence. It is particularly advantageous here to directly use the fluorescence change for the analysis and to infer a methylation state from the fluorescence signal. A particularly preferred variant is the "Taqman" method. In another particularly preferred embodiment, an additional fluorescence-marked oligomer is used, which hybridizes in immediate proximity to the first reporter oligonucleotide, and this hybridization can be detected by means of fluorescence resonance energy transfer ("Lightcycler" method).

It is a preferred embodiment of the invention to amplify several fragments at the same time by means of a multiplex PCR. Care has to be taken when designing that not only the primers, but also the other oligonucleotides used must not be complementary to each other, so that a high-degree multiplexing is more difficult in this case than usual. It is aggravating, further, that the bisulphite-caused conversion of the nucleic acids reduces the complexity thereof. However, the chemically pretreated DNA offers the advantage that due to the differing G and C contents of the two DNA strands, a forward primer can never act as a reverse primer, too, which again facilitates the multiplexing and substantially compensates the disadvantage described above. The detection of the amplificates is in turn possible by different methods. The use of real time methods is for instance imaginable. For amplifications of more than four genes, it is however recommendable to detect the amplificates in a different way. An analysis by means of microarrays (see above) is preferred.

An updated survey of further possible methods

for the methylation analysis is found in: Fraga and Esteller 2002, ibid.).

In the different methods for the methylation analysis, the MDA-DNA can be used as a standard in different ways. A standard is therein on the one hand any kind of negative control or positive control in the case of the detection of non-methylated DNA. This is in particular the case for technologies, which detect smallest amounts of methylated DNA in a big background of non-methylated DNA and vice versa. This case is also called "sensitive detection". Therein, the non-methylated MDA-DNA serves during the assay development as a verification of the specificity of the assay for methylated DNA and during the application of the assay as a negative control. It is however also preferred according to the invention to use a mixture of non-methylated DNA and methylated DNA. It is particularly preferred to use different mixtures (i.e. consisting of different shares) of non-methylated and methylated DNA. Thereby, then calibration curves can be prepared. In order to prepare these mixtures, preferably the non-methylated DNA produced by MDA is used as a base. The total amount of the control DNA is subdivided and a part thereof is methylated by means of an SssI methylase (see above). The other part of the non-methylated DNA is also reacted with all reaction components of the methylation batch, except for the methylase. Thus it is secured that the DNA concentration in both batches is identical, and that in both batches the same reaction components are present. Subsequently, non-methylated and methylated DNA are mixed in different ratios, for instance in a ratio 4:0 for 0%, 3:1 for 25%, 2:2 for 50%, 1:3 for 75%, 0:4 for 100%. For the de-

velopment of assays for the sensitive detection, it may be preferred to produce mixtures with very small concentrations of methylated DNA (for instance 1:2,000 - 1:10,000).

By calculating the quotient of the signals, which are detected for the methylated state, and the signals, which are detected for the non-methylated state, the measured methylation rate is obtained. If this is plotted against the theoretical methylation rates (according to the share of methylated DNA in the defined mixtures), and the regression through the measured points is determined, a calibration curve is obtained. By using this calibration curve, the methylation level of the unknown samples can be determined by means of the measured methylation rate.

The verifications or standards described further above can be used for all methods of the quantitative methylation analysis: amongst others, for MS-SNuPE, for the hybridization on microarrays, hybridization assays in solution, direct bisulphite sequentiation, for real time PCR (e.g. HeavyMethyl, MSP. comp. for the PMR values: Eads et al., CANCER RESEARCH 61, 3410-3418, April 15, 2001).

A particularly preferred use of the DNA produced by WGA methods and of the method according to the invention is the diagnosis of cancer diseases or other diseases associated with a modification of the methylation state. Thereto belong amongst others, CNS malfunctions, aggression symptoms or conduct disorders; clinical, psychological and social consequences of brain damages; psychotic disorders and personality

disorders; dementia and/or associated syndromes; cardiovascular disease, malfunction and damages; malfunction, damages or disease of the gastrointestinal tract; malfunction, damages or disease of the breathing system; injury, inflammation, infection, immunity and/or convalescence; malfunction, damages or disease of the body as a deviation in the development process; malfunction, damages or disease of the skin, of the muscles, of the connective tissue, or the bones; endocrine and metabolic malfunction, damages or disease; headaches or sexual malfunction. The method according to the invention is further suitable for the prognosis of undesired effects of drugs and for the differentiation of cell types or tissues or for the investigation of the cell differentiation.

According to the invention is furthermore provided a kit composed of reagents for performing a WGA method or of DNA amplified already by a WGA method and of reagents for performing a bisulphite conversion, and optionally also contains a polymerase, primers and/or probes for an amplification and detection.

According to the invention is further provided fully methylated DNA, which has been produced by a WGA method and then methylated by means of an enzyme, preferably the SssI methylase. According to the invention is finally also provided a mixture of methylated and non-methylated DNA produced by a genome-wide amplification method. Such a mixture can particularly be used as a standard for methylation analysis. The production of the mixture can thereby be carried out as described above. Preferred are mixtures with a portion of between 5 and 95% of methylated DNA, particularly preferred are

mixtures with a portion of between 10 and 80% of methylated DNA, exceptionally preferred are mixtures with a portion of between 25 and 75% of methylated DNA.

Example:

use of MDA-DNA for calibrations.

The methylation degree of a DNA from abdominal fatty tissue is to be determined by means of oligonucleotide microarrays and for a comparison by means of the MS-SNuPE method. For this purpose, the DNA is extracted from the biological sample by means of the QIAamp Mini Kit (Qiagen) according to manufacturer's instructions. For determining a calibration curve, different mixtures of methylated and non-methylated DNA are produced (0%, 25%, 50%, 75%, 100% methylated DNA). The non-methylated DNA was obtained from Molecular Staging, where it has been produced by an MDA reaction from human genomic DNA of full blood. In an MDA reaction, all methylation signals are deleted (see above). The fully methylated DNA is produced from the MDA-DNA by means of an SssI methylase (New England Biolabs). The synthesis is performed according to manufacturer's instructions. The remaining non-methylated DNA is reacted with all reagents except for the SssI methylase. Thus it is secured that the DNA concentration is identical in both batches, and that in both batches the same reaction components are present. Then, non-methylated and methylated DNA are mixed in the following ratios: 4:0 for 0%, 3:1 for 25%, 2:2 for 50%, 1:3 for 75%, 0:4 for 100%. The DNA is then bisulphite-converted in presence of dioxane as a

denaturating solvent (cf.: DE 10029 915 Al; German application: File No.: 10347396.3). Subsequently, the prepared DNA mixtures and the DNA from the biological sample are employed in a multiplex PCR. 8 fragments each are amplified. As primers are used the oligonucleotides listed in Table 1. The amplifications are performed by means of the QIAGEN HotStarTaq Kit essentially according to manufacturer's instructions and with the following temperature profile: 95°C: 15 min; 45 times: (95°C: 15 sec; 55°C: 30 sec; 72°C: 60 sec); 72°C: 10 min. The multiplex PCR products are then hybridized at an oligomer microarray. The probe oligonucleotides are listed in Table 2. The hybridization and the methylation signal determination are made as described by Adorjan et al., 2002 (ibid.). For each sample and each calibration mix, eight hybridizations are performed. For the preparation of calibration curves for a CpG position, the measured methylation rate is plotted against the theoretical methylation rate. The measured methylation rate results from the signal intensity of an oligonucleotide probe, which is specific for the methylated state, divided by the total intensity of this probe + a matching (i.e. covering the same CpG position) probe, which is specific for the non-methylated state. The theoretical methylation state corresponds to the methylation levels of the used defined mixtures. Oligonucleotide probe pairs, which are suitable for calibration purposes, have monotonously increasing calibration curves. For the Ms-SNuPE reaction, the samples are amplified with the primers mentioned above in individual PCR reactions. The reaction conditions are the same as for the multiplex PCR (see above). In the extension reaction, positions are used as primer binding

sites, which are positioned directly at the flanks of CpG positions, which correspond to those of the oligonucleotide microarrays. The Ms-SNuPE assay is performed according to the instructions of the manufacturer of the MegaBace-SNuPE kit. For the two possible variants of the nucleotides to be incorporated, ddNTP's marked with different dyes are used. For every SNuPE assay, four measurements are made in parallel. The signal intensities determined by the SNP profile software (Amersham) (Imeth- for non-methylated specific probes and Imeth+ for methylated specific probes) of the two employed dyes are used according to the quotient Imeth+/(Imeth- + Imeth+), in order to determine the measured methylation rate. By plotting these values against the theoretical methylation rate, again a calibration curve is obtained, which should be monotonously increasing. The monotonously increasing calibration curves thus generated are used to determine the actual methylation from the measured methylation rate of sample DNA.The results are shown in Fig. 1. The y-axis represents the percentage of methylation, the x-axis shows the hybridization at different oligonucleotides or different SNuPE assays.The methylation rates in sample DNA determined by the two methods corrected at corresponding calibration curves are in a good agreement for the shown CpG positions. These data show that non-methylated DNA produced by MDA or corresponding mixtures with methylated DNA can be used very well as a standard in the methylation analysis.

Table 1: Primer for the Multiplex-Amplification

(Please refer to the claimed priority application no.: EP 04090037.5, filed February 05, 2004)

Table 2: oligonucleotides

(Please refer to the claimed priority application no.: EP 04090037.5, filed February 05, 2004)

Patent claims.

1. A method for the methylation analysis, characterized by that

c) a genome-wide amplification is performed,

d) the amplificates generated in a) are used as a standard in the methylation analysis.

2. The use of DNA produced by genome-wide amplification methods as a standard in the methylation analysis.

3. A method or the use according to claim 1 or 2, characterized by that PEP, DOP-PCR or linker PCR are performed as an amplification method.

4. A method or the use according to claim 1 or 2, characterized by that a multiple displacement amplification (MDA) is performed as an amplification method.

5. A method or the use according to claim 4, characterized by that a φ29 polymerase is used in the MDA.

6. A method or the use according to claim 4, characterized by that the MDA is performed by means of a commercially available kit.

7. A method or the use according to claim 6, characterized by that "GenomiPhi" (Amersham Biosciences) or "Repli-g" (Molecular Staging) is used as a kit.

8. A method or the use according to claim 4, characterized by that commercially available DNA produced by MDA is used as a standard.

9. A method or the use according to at least one of claims 1 - 8, characterized by that the methylation analysis is performed by restriction enzymes.

10. A method or the use according to at least one of claims 1 - 8, characterized by that the methylation analysis is performed by methylation-specific ligation methods, MSP, Heavy Methyl or MethyLight.

11. A method or the use according to at least one of claims 1 - 8, characterized by that the methylation analysis is performed by primer extension.

12. A method or the use according to at least one of claims 1 - 11, characterized by that the methylation analysis is performed by an amplification and a hybridization of the amplificates at oligomer microarrays.

13. A method or the use according to at

least one of claims 1 - 12, characterized by that the methylation analysis is performed by means of a multiplex PCR.

14. A method or the use according to at least one of claims 1 - 13, characterized by that a mixture of methylated and non-methylated DNA is used as a standard.

15. A method or the use according to at least one of claims 1 - 14, characterized by that several mixtures of methylated and non-methylated DNA with different shares of methylated and non-methylated DNA are used as a standard.

16. A method or the use according to at least one of claims 1 - 15, characterized by that the methylation analysis is performed for the diagnosis of cancer diseases or other diseases associated with a modification of the methylation status.

17. A method or the use according to at least one of claims 1 - 16, characterized by that the methylation analysis is performed for the prognosis of desired or undesired effects of drugs and for the differentiation of cell types or tissues, or for the investigation of the cell differentiation.

18. A kit comprising reagents for performing a WGA method or DNA amplified already by a

WGA method and reagents for performing a bisulphite conversion, and optionally also containing a polymerase, primers and/or probes for an amplification and detection.

19. A methylated DNA produced by a WGA method and then methylated by means of an enzyme.

20. A methylated DNA produced by a WGA method and then methylated by means of the SssI methylase.

21. A mixture of methylated and non-methylated DNA produced by a genome-wide amplification method.

22. A mixture of methylated and non-methylated DNA produced by a genome-wide amplification method, wherein the share of methylated DNA is between 5 and 95%.

23. A mixture of methylated and non-methylated DNA produced by a genome-wide amplification method, wherein the share of methylated DNA is between 10 and 80%.

24. A mixture of methylated and non-methylated DNA produced by a genome-wide amplification method, wherein the share of methylated DNA is between 25 and 75%.

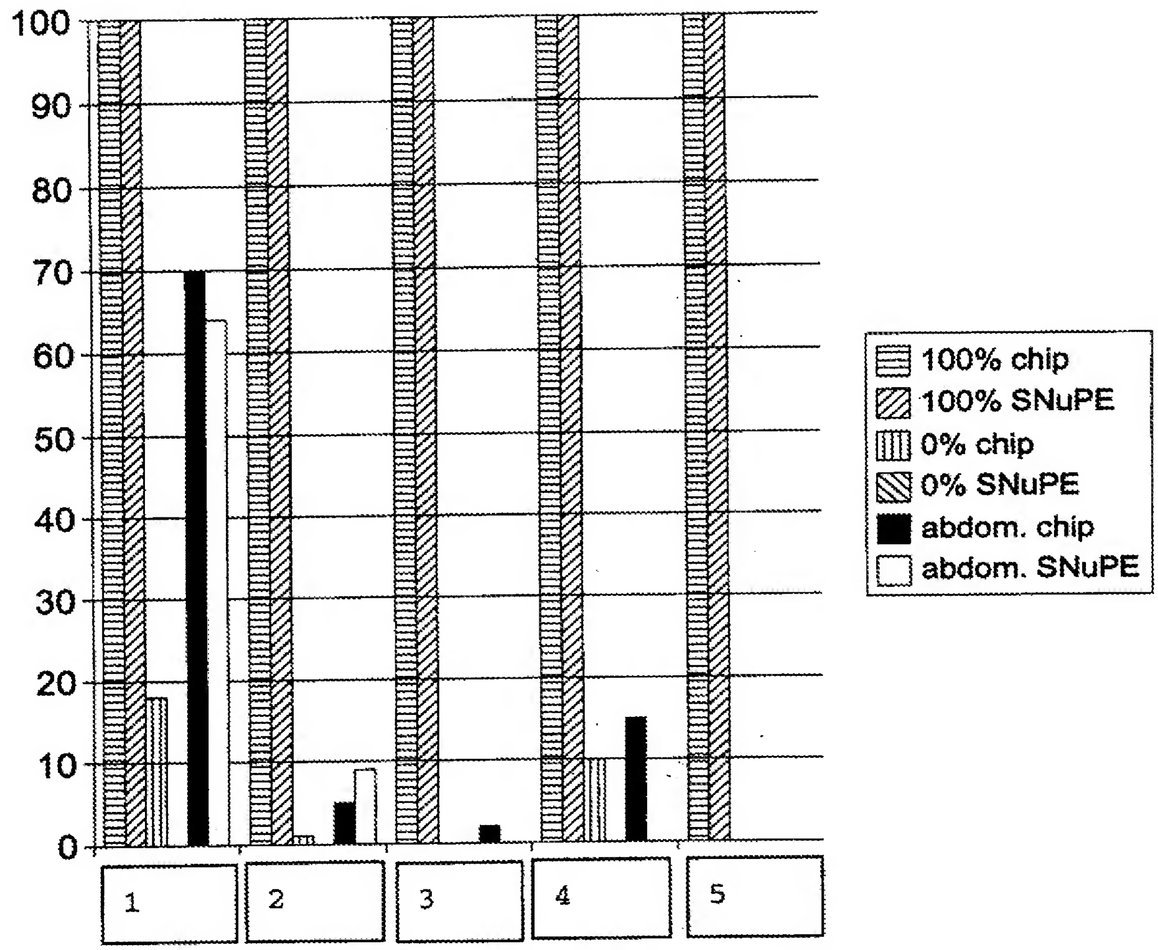
25. The use of the DNA according to claims 19 to 20 or of a mixture according to claims 21 to 24 for the methylation analysis.

Abstract.

The present invention relates to a method for the production of DNA, in which 5-methylcytosine is not present. Such non-methylated DNA is in particular required as a verification for a reliable and sensitive analysis of cytosine methylations. The non-methylated DNA is synthesized by genome-wide amplification methods, in particular by a multiple displacement amplification (MDA). The non-methylated DNA can be used as a standard in a multitude of methods for the methylation analysis.

Sequence Listings

(Please refer to the claimed priority application no.: EP 04090037.5, filed February 05, 2004)


100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
100% chip
100% SNuPE
0% chip
0% SNuPE
abdom. chip
abdom. SNuPE